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Increased prevalence of antibodies to enteropathogenic *Yersinia enterocolitica* virulence proteins in relatives of patients with autoimmune thyroid disease

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SUMMARY

Infections have been implicated in the pathogenesis of a number of autoimmune diseases, and *Yersinia enterocolitica* (YE) might play a role in the development of autoimmune thyroid disease (AITD). Clinical evidence in support of this hypothesis has been inconclusive. We reasoned that looking earlier in the natural course of AITD might enhance chances of finding evidence for YE infection. Consequently, we determined seroreactivity against YE in subjects at risk of developing AITD, i.e. in 803 female relatives of AITD patients in self-proclaimed good health. As a comparison group we used 100 healthy women who participated in a program for reference values. IgG and IgA antibodies to virulence-associated outer membrane proteins (YOPs) of YE were measured by a specific assay. Serum thyroid peroxidase antibodies (TPO-Ab) as indicators of AITD were considered to be positive at levels of >100 kU/l. The prevalence of YOP IgG-Ab was higher in AITD relatives than in controls (40·1% vs. 24%, $P = 0{\cdot}002$), and the same was true for YOP IgA-Ab (22% vs. 13%, $P < 0{\cdot}05$). Of the 803 AITD relatives, 44 had an increased or decreased plasma TSH, and 759 were euthyroid as evident from a normal TSH; the prevalence of YOP-Ab did not differ between these three subgroups. TPO-Ab were present in 10% of controls and in 27% of the AITD relatives ($P < 0{\cdot}001$). The prevalence of TPO-Ab in the euthyroid AITD relatives was not different between YOP IgG-Ab positive and negative subjects (23·3% vs. 24·7%, NS), nor between YOP IgA-Ab positive and negative subjects (21·2% vs. 24·9%, NS). In conclusion, healthy female relatives of AITD patients have an increased prevalence of YOP antibodies, which, however, is not related to the higher prevalence of TPO antibodies in these subjects. The findings suggest a higher rate of persistent YE infection in AITD relatives. Susceptibility genes for AITD may also confer a risk for YE infection.

Keywords autoimmune thyroid disease family *Yersinia enterocolitica* thyroid peroxidase

INTRODUCTION

Autoimmune thyroid disease (AITD) is viewed as a multifactorial condition in which the development of the autoimmune response against thyroidal antigens is facilitated by a particular, but still incompletely known, polygenetic background and presumably provoked by environmental factors. Identified environmental factors are iodine intake, smoking, stress, pregnancy, oestrogens and possibly infections. Infections have been implicated in the pathogenesis of a number of autoimmune diseases like Coxsackievirus P2-C in type 1 diabetes mellitus [1], but their role in AITD is disputed. The best-studied infection in AITD is that with *Yersinia enterocolitica* (YE). The membrane of YE has specific TSH binding sites [2], and infection with YE gives rise to antibodies against these TSH binding sites which recognize and stimulate the TSH receptor of human thyroid membranes [3,4]. Conversely, Graves IgG bind to YE membranes [5]. Thus, YE infection by molecular mimicry with self antigens may induce cross-reactive TSH receptor antibodies and cross-reactive T-cells, leading to AITD. YE furthermore acts as a superantigen [6], and may result, via induction of V-gene restricted T-cells, in polyclonal stimulation of autoreactive T-cells, again contributing to the development of AITD.

Whereas the laboratory data provide a solid base for assuming an aetiologic role of YE infection in AITD, clinical evidence in support of this hypothesis has been inconclusive, as conflicting results are reported on the frequency of YE infection in AITD patients compared with controls, as summarized in Table 1 [7–12]. How are these discrepant results explained? Geographical differences in exposure to YE might be one reason; differences in the

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Table 1. Summary of literature data on the frequency of *Yersinia enterocolitica* (YE) infection in patients with autoimmune thyroid disease, compared with controls

Reference	Country	Applied method for YE seroreactivity		Frequency of YE infection		
				Graves' disease	Hashimoto's disease	Controls
[7]	Germany	Western blot	IgG	60–81%*	66%*	35%
			IgA	29–39%*	37%*	12%
[8]	Greece	ELISA	IgG	–	25·4%*	1·9%
			IgA	–	2·8%*	0%
[9]	USA	Western blot	IgG	96%	55·5%	70·8%
[10]	Canada	Western blot	IgG	>90%	>90%	>90%
		ELISA	IgG	Not different from controls		
		Agglutination	O3, O9	All –ve	All –ve	All –ve
[11]	Japan	Agglutination	O3	27·1%	40·0%	29·4%
			O5	81·4%*	91·1%*	58·9%
			O6	28·6%	40·0%*	24·7%
			O9	37·1%	51·1%*	29·9%
[12]	Turkey	Agglutination	O3	53·8%*	38·1%*	17·5%
			O5	29·2%*	15·8%*	7·5%
			O8	44·6%*	17·5%*	7·5%
			O9	40·0%*	22·8%*	10·0%

*Significantly different from controls.

applied methods to measure YE seroreactivity could be another. Serologic evidence of YE infection can be obtained by the agglutination reaction, which however, becomes rapidly negative. All pathogenic Yersinia species harbour a 70-kb plasmid encoding for the virulence conferring outer membrane proteins (YOPs) [13,14]. A more suitable method, therefore, is the demonstration of specific IgA and IgG antibodies against YOPs by ELISA or immunoblots. Methodological differences, however, are unlikely to be accountable for the sharp contrast between positive and negative studies when similar methods are used. A third possibility is that the studies were carried out too late in the course of the disease. Indeed, all reported studies in the literature are cross-sectional in nature, investigating serum samples of patients who already had developed full blown Graves' hyperthyroidism or Hashimoto's hypothyroidism and who had mostly been treated for months to years. Although it has been reported that IgA and IgG antibodies in newly diagnosed patients with Graves' hyperthyroidism are not observed until 4 weeks after diagnosis [15], patients rarely remember any symptoms of a recent Yersinia infection [8]. We hypothesized that looking earlier in the natural course of the disease (i.e. when thyroid function is still normal but thyroid antibodies are already present) might increase the chances of finding evidence of YE infection. This is because IgA antibodies appear after 10 days post infection, followed by IgG antibodies. IgA reactivity decreases rapidly after 3–6 months, whereas a decrease of IgG is markedly retarded. In chronic infection, persistent IgA and IgG reactivity is seen [13].

To pursue this further, the question is how to find subjects with subclinical AITD. Female relatives of patients with Graves' hyperthyroidism or Hashimoto's hypothyroidism are clearly at risk in developing AITD in view of their gender and family history [16,17]. We have assembled a large group of such subjects in the Amsterdam AITD cohort study, designed as a long-term prospective follow-up study to get more insight into genetic and environmental factors involved in the pathogenesis of AITD [18]. Here we report the results at study entrance for YE seroreactivity, which was assayed blindly with respect to the thyroid state. As a comparison, we recruited healthy euthyroid volunteers. Serum samples of both groups were collected over the same period of time.

SUBJECTS AND METHODS

The Amsterdam AITD cohort comprises 803 female subjects between 18 and 65 years of age with at least one 1st- or 2nd-degree relative with documented autoimmune hyper- or hypothyroidism; they had no personal history of thyroid disease and were in self-proclaimed good health. All subjects were seen at our institution, and blood was drawn after obtaining informed consent; plasma and serum samples were stored at –20°C until assay.

As a comparison group, we used 100 female subjects between 20 and 69 years of age, who were recruited through advertisements in local newspapers, to participate in an ongoing programme with our institution for delineating reference values of endocrine function tests. They were also in self-proclaimed good health, and had no history of thyroid disease. Blood samples were collected over the same period of time as those of the Amsterdam AITD cohort, and processed in the same manner. The study was approved by the institutional Committee on medical ethics in Amsterdam.

In all subjects free thyroxine (FT4, time-resolved fluoroimmunoassay, Delfia, Turku, Finland) and thyrotropin (TSH, Delfia) were measured, as well as antibodies against thyroid peroxidase (TPO-Ab, chemiluminescence immunoassay, LUMI-test, Brahms, Berlin, Germany). Reference values of FT4 are 9·3–20·1 pmol/l and of TSH 0·4–5·7 mU/l. TPO-Ab were considered to be positive at levels of >100 kU/l; interassay variation at 70 kU/l is 15%, and at 600 kU/l 5·7%.

Table 2. Prevalence of antibodies against thyroid peroxidase (TPO-Ab) and against virulence-associated YOPs of *Yersinia enterocolitica* (YOP IgG-Ab and YOP IgA-Ab) in sera of 803 female relatives of patients with autoimmune thyroid disease (AITD) and 100 healthy female controls

	n	Age	TPO-Ab	YOP IgG-Ab	YOP IgA-Ab
Controls	100	44 ± 15	10 (10%)	24 (24%)	13 (13%)
AITD relatives	803	36 ± 12	216 (27%)‡	322 (40·1%)*	176 (22%**)
AITD relatives					
Hypothyroid	29	43 ± 12	25 (86%)	10 (34·5%)	7 (24·1%)
Euthyroid	759	36 ± 12	183 (24%)‡	305 (40·2%)*	165 (21·7%)**
Hyperthyroid	15	44 ± 10	8 (53%)	7 (46·7%)	4 (26·7%)

‡$P < 0{\cdot}001$, *$P = 0{\cdot}002$, **$P < 0{\cdot}05$ vs. controls.

Table 3. Prevalence per age group of antibodies against virulence-associated YOPs of *Yersinia enterocolitica* (YOP IgG-Ab and YOP IgA-Ab) in sera of 803 female relatives of patients with autoimmune thyroid disease

Age	*n*	YOP IgG-Ab *n* (%)	YOP IgA-Ab *n* (%)
18–29 year	295	123 (41·7%)	69 (23·4%)
30–39 year	215	88 (40·9%)	45 (20·9%)
40–49 year	170	64 (37·6%)	36 (21·2%)
50–59 year	94	36 (38·3%)	18 (19·1%)
60–69 year	29	11 (37·9%)	8 (27·6%)

Table 4. Characteristics of 759 euthyroid healthy female relatives of patients with autoimmune thyroid disease according to the presence or absence in serum of antibodies against virulence-associated YOPs of *Yersinia enterocolitica*

	n	Age year (x ± s.d.)	TSH (mU/l)*	TPO-Ab > 100 kU/l *n* (%)
YOP IgG-Ab				
Positive	305	35 ± 12	1·6 (1·2–2·3)	71 (23·3%)
Negative	454	36 ± 12	1·8 (1·3–2·5)	112 (24·7%)
YOP IgA-Ab				
Positive	165	35 ± 12	1·8 (1·2–2·3)	35 (21·2%)
Negative	594	35 ± 12	1·7 (1·2–2·5)	148 (24·9%)

*Median and interquartile range.

Specific IgG and IgA antibodies against purified plasmid-encoded virulence associated YOPs of YE serotype O9 (LCR) in sera were demonstrated by immunoblotting with a YOP-Ab assay (AID, Strassberg, Germany). In short, antigens (25, 34, 36, 37, 39, 40, 46, 48 kDa) are blotted onto nitrocellulose. Sera are diluted 1 : 51 in PBS-Tween and incubated with the antigen-coated nitrocellulose strips overnight at 22°C. The IgG and IgA antibody-antigen complexes formed are quantified after immunostaining with the AID-Scan-System. Controls are included in each assay run, using human acute sera (culture-positive YE infection) containing antibodies to the YOPs. Test sera are judged positive if at least three bands (IgG) or two bands (IgA) are seen in immunoblotting at a level greater than 10% (IgG) or 5% (IgA) of reference standards. The interassay variation of the YOP-Ab assay is <3% according to the manufacturer. The YOP-Ab assay was performed without prior knowledge of thyroid function tests or the presence of TPO-Ab in the serum samples.

The significance of differences between groups was analysed with the X^2 test, or with Fisher exact test in the case of small numbers. *P*-values are two tailed.

RESULTS

The prevalence of TPO antibodies and of IgG and IgA antibodies against YOPs was higher in AITD relatives than in controls (Table 2). The AITD relatives were, on average, nine years younger than the control women, but the higher prevalence of YOP antibodies in AITD relatives was similar in all age groups (Table 3).

The majority (94·5%) of AITD relatives were euthyroid, as evident from a normal TSH, but 3·6% were hypothyroid (plasma TSH > 5·7 mU/l) and 1·9% were hyperthyroid (plasma TSH < 0·4 mU/l). The prevalence of YOP antibodies did not differ between the euthyroid, hypothyroid and hyperthyroid AITD relatives (Table 2).

The relative risk in AITD female relatives is 1·57 (95% CI 1·09–2·25) for the presence of YOP IgG-Ab, and 1·68 (95% CI 1·00–2·84 for YOP IgA-Ab. The relative risk in the subgroup of euthyroid AITD relatives is similar: 1·67 (95% CI 1·17–2·40) for YOP IgG-Ab, and 1·67 (95% CI 0·99–2·83) for YOP IgA-Ab. Next, we examined the determinants of the presence of YOP antibodies in the subgroup of euthyroid AITD relatives (Table 4). Subjects with YOP IgG-Ab were not different from those without YOP IgG-Ab with respect to age, plasma TSH and prevalence of TPO antibodies. The same was true for YOP IgA-Ab. The presence of YOP IgG-Ab and IgA-Ab was also not related to smoking behaviour, oestrogen medication, parity or a history of iodine excess (data not shown). Two of the euthyroid AITD relatives had TSH-binding inhibitory immunoglobulins (TBII) in their serum, but YOP IgG and IgA antibodies were absent in both.

DISCUSSION

The main finding of the present paper is a higher prevalence of IgG and IgA antibodies to released virulence-associated outer membrane proteins (YOPs) of *Yersinia enterocolitica* in female relatives of patients with autoimmune thyroid disease than in control women. The validity of this finding depends heavily on the

characteristics of the control group: are these comparable with the AITD relatives? All investigated subjects were in self-proclaimed good health and of female sex, but the control women were, on average, eight years older. However, the difference in age is unlikely to account for the difference of 16·1% (95% CI 16·0–16·2) in the prevalence of YOP IgG-Ab between both groups, nor for the difference of 9% (95% CI 8·9–9·1) in the prevalence of YOP IgA-Ab, because the presence of YOP antibodies was similar in all age groups. Blood samples of both groups were collected over the same time period, excluding bias due to seasonal variation in Yersinia infection. Control women orginated from Amsterdam and surrounding areas, but AITD relatives came from all over the Netherlands. Confounding bias due to some variation in places of residence seems unlikely as no specific geographical distribution of Yersinia infections has been reported in the Netherlands. Measurement bias can be ruled out because all assays of YOP antibodies were done 'blindly' using one particular method. The groups differed in thyroid function: control women were all euthyroid, but 5·5% of the female AITD relatives were either hypo- or hyperthyroid. However, thyroid function by itself does not explain the difference in YOP antibodies between both groups: the prevalence of YOP antibodies was equally high in hypo-, hyper- and euthyroid AITD relatives, and the difference in prevalence of YOP antibodies between euthyroid AITD relatives and controls was the same as between the whole group of AITD relatives and controls.

Can the higher prevalence of YOP antibodies in AITD relatives be explained from the difference in prevalence of AITD between both groups? The presence of serum TPO antibodies is a good marker for the existence of chronic autoimmune thyroiditis [19]. According to this criterion, AITD was present in 10% of control women, a figure in good agreement with the prevalence of AITD in the adult female population [20]. As expected, we found a much higher prevalence of AITD of 27% in the female AITD relatives. Nevertheless, the difference in prevalence of AITD between both groups cannot be held accountable for the difference in prevalence of YOP antibodies, as the presence of YOP antibodies in euthyroid relatives was not related at all to the presence of TPO antibodies. One may argue that our cut-off value of >100 kU/l in the TPO-Ab assay as criterion for a positive TPO-Ab test is too high because the detection limit of the assay is 50 kU/l, and values between 50 and 100 kU/l were observed in 103 out of the 759 euthyroid AITD relatives. Although the biologic significance of these intermediate values is less well established, accepting all values greater than 50 kU/l as a positive TPO-Ab test result does not alter the picture: the prevalence of TPO-Ab in the YOP IgG-Ab positive and IgG-Ab negative subjects is then 35·1% and 39·4%, respectively (not significantly different), and in the YOP IgA-Ab positive and IgA-Ab negative subjects 29·7% and 39·9% ($P = 0{\cdot}017$). These figures show an even lower frequency of TPO-Ab in YOP IgA-Ab-positive than in IgA-Ab-negative subjects. Our findings led us to conclude that the higher prevalence of YOP antibodies in AITD relatives is not related to the higher prevalence of AITD in these subjects.

The lack of association between seroreactivity against YE and AITD argues against Yersinia infection as a causal factor contributing to the development of AITD. We found no support for our initial hypothesis that chances to observe such an association would be enhanced by looking earlier in the natural course of AITD, i.e. when thyroid function is still normal but thyroid antibodies are already present. The limitations of the present study are, however, the same as of those reported so far in the literature: they are all cross-sectional in nature. It cannot be completely excluded that during prospective long-term follow-up, as foreseen in our Amsterdam AITD cohort study, the occurrence of TPO antibodies or an abnormal thyroid function bears a temporal relationship to a previous rise of YOP antibodies. Such an observation would strengthen a cause-and-effect relationship.

Having ascertained the validity of the comparison group and the lack of an association between YOP antibodies and AITD, how must the higher prevalence of YOP antibodies in AITD relatives be interpreted? Yersinia virulence plasmids encoding for YOPs have not been found in other enterobacteriacaea. The measured YOP antibodies are apparently specific for YE, and a fair proportion of our investigated subjects must have experienced YE infection. Indeed, Yersiniosis seems a rather common disease in the Netherlands [13]. The lower prevalence of YOP IgA-Ab relative to IgG-Ab indicates that YE infection in our adult subjects must have occurred somewhere in the past and not in recent times. In line with this are demographic data of 261 Dutch patients with enteric forms of Yersiniosis [13]: 40·2% had occurred in the age group of 0–15 years, 18% between 16 and 25 years, and 41·8% in patients older than 25 years.

Our data do not necessarily indicate a higher incidence of YE infection in AITD relatives. This is because, in 65% of patients with yersiniosis, the infection is self-limiting, but in 35% a chronic infection persists with high titres of YOP IgG-Ab and IgA-Ab due to continuous antigenic stimulation from the plaques of Peyer and other lymph nodes still harbouring YE [21]. Why YE infection may persist in some subjects but is self-limiting in others must be determined by host factors. Experimental animal models suggest that the immune state is an important determinant [22]. In human and in mice, a relationship has been noted between the infection pattern and the HLA-B27 antigen [23,24]. Following this line of reasoning, it could be that the higher prevalence of YOP antibodies in AITD relatives is not caused by a higher incidence of YE infection *per se*, but by a higher rate of persistent YE infection caused by a particular genetic make-up. Some of the susceptibility genes for AITD (like those of the HLA family and CTLA-4) may also confer a risk for persistence of YE infection. For, unlike individuals constantly exposed to infections, individuals living under hygienic conditions (as in the Netherlands) are poor regulators of immune responses in general. According to the 'hygiene hypothesis' the decreased infections and antigenic pressure of a westernized lifestyle may result in an increased incidence of allergic and organ-specific autoimmune diseases [25,26]. We consider this possibility as the most attractive explanation of our results.

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